Docket No.: 00131-00350-US
(PATENT)

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Patent Application of:
Ann S. Robinson et al.

Application No.: 10/673000

Confirmation No.: 9773

Filed: September 26, 2003

Art Unit: 1639

For: USE OF HYDROSTATIC PRESSURE TO INHIBIT AND REVERSE PROTEIN AGGREGATION AND FACILITATE PROTEIN REFOLDING

Examiner: M. C. T. Tran

DECLARATION UNDER 37 C.F.R. § 1.132

MS Amendment
Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

Dear Sir:

1. I, Anne Skaja Robinson declare as follows:
2. I am currently an associate professor of chemical engineering at the University of Delaware and I am an inventor named on the above-referenced patent application. Attached as **Exhibit 38** is my current CV.
3. I have read the Office Action dated June 30, 2006 in the above referenced patent application.
4. I disagree with the Examiner's contention that United States Application 09/695,762, to which the above referenced application claims priority, fails to disclose the subject matter claimed in claims 3-6, 8 and 10-12. In particular I note that:

a. The "Summary of the Invention" at page 2, lines 20-28, the 09/695,762 application teaches:

> It has been discovered that by application of hydrostatic pressure, protein aggregation can be inhibited or reversed. It is effective both in preventing aggregation during refolding and in reversing aggregation which has already taken place. After pressure is released, dissociated aggregates refold to form biologically active protein with native characteristics. ...
>
> An additional benefit of the present invention is that the **use of the present invention substantially** or even entirely obviates the need for urea or other denaturants.

(Emphasis added).

b. The summary of the invention, the 09/695,762 at page 4, lines 1-16, further states in relevant part that:

> The present invention also provides a method to inhibit or reverse protein aggregation by subjecting a sample to high hydrostatic pressure, where the sample contains a protein aggregate, so as to substantially unfold the protein of the protein aggregate, and returning the sample to ambient pressure so as to allow the unfolded protein to refold, thereby recovering native protein from the protein aggregate. ....
>
> The invention also provides **embodiment of such methods where the sample is substantially free of a denaturing agent** selected from the group consisting of guanidine hydrochloride, guanidine thiocyanate, sodium dodecyl sulfate (SDS), and Urea. Similarly the present invention provides embodiments of such methods where the sample is substantially free of sodium dodecyl sulfate (SDS).
>
> .... Similarly, the invention provides embodiments of such methods **where the denatured protein is unfolded in the presence of a reducing agent**.

(Emphasis added).

c. One skilled in the art would necessarily understand the above passages of the 09/695,762 application as indicating that some methods of the invention involve use of protein aggregates in a sample further containing "a chaotropic agent in an amount

which is insufficient to denature said native protein at ambient pressure," as that phrase is used in claims 4 and 10 of the above referenced application. Specifically, by definition a denatured protein does not have the same conformation as the native protein. It necessarily follows that in those embodiments of the invention which use as a starting material a sample comprising protein aggregates and a chaotropic agent, that it would not be possible to recover native protein at ambient pressure following the hydrostatic pressurization step if a chaotropic agent were present in an amount sufficient to denature the protein at ambient pressure.

5. I disagree with the Examiner's contention that United States Application 09/695,762, to which the above referenced application claims priority, fails to disclose the subject matter claimed in claims 3, 5, 8 and 11, which all call for application of a pressure insufficient to fully denature the protein. In particular I note that:

a. The "Summary of the Invention" of 09/695,762 at page 4, lines 1-7, states that:

> The present invention also provides a method to inhibit or reverse protein aggregation by subjecting a sample to high hydrostatic pressure, where the sample contains a protein aggregate, so as to substantially unfold the protein of the protein aggregate, and returning the sample to ambient pressure so as to allow the unfolded protein to refold, thereby recovering native protein from the protein aggregate. In accordance with certain embodiment of such methods, the high hydrostatic pressure is from preferably about 1 to about 3.5 kbar or about 2.5 kbar.

b. In the paragraph spanning pages 6-7 of 09/695,762 states in relevant part that:

> It has been discovered that by application of hydrostatic pressure, protein aggregation can be inhibited or reversed. .... After pressure is released, dissociated aggregates refold to form biologically active protein with native characteristics. .... The partially unfolded intermediates appear to preferably refold into the conformation of the native protein, rather than merely re-aggregating upon release of pressure.

c. As indicated at page 6, line 18 – page 7, line 3 of 09/695,762, it was well known in art that application of hydrostatic pressure to proteins can be carried out without denaturing the tertiary and/or secondary structure of proteins. In particular, as noted at page 7, lines 1-3 of 09/695,762 (citing references), "secondary and tertiary structures of proteins typically do not denature until pressures above 5 kbar."

d. Finally, at page 9, lines 3-12 of 09/695,762, it is stated that:

> Reversal of protein aggregation by the present invention is believed to be somewhat analogous to pressure dissociation of oligomeric proteins. **The chains that are dissociated by pressure are competent for rapid productive folding, perhaps because the secondary and tertiary structure is preserved.** The pressure-sensitive interfaces of aggregates are likely to be well-packed and solvent-excluded, suggesting that aggregation involves specific protein-protein interactions.
>
> In accordance with the present invention, a sample containing a protein of interest is subjected to high hydrostatic pressure. Preferably, the hydrostatic pressure is between about 0.5 kbar and 10 kbar, preferably about 1 kbar to about 3.5 kbar, most preferably about 2 to 3 kbar.

(Emphasis added.)

e. Based on the knowledge in the art as to the level of pressure required to denature the secondary and tertiary structures of proteins (i.e., above 5 kbar), one skilled in skilled in the art would immediately appreciate that the range of hydrostatic pressures applied in some preferred embodiments (i.e., 1-3.5 kbar) would be "insufficient to fully denature said protein" or "said protein folding intermediates."

f. Furthermore, as highlighted in the passage from 09/695,762 cited in paragraph d., the present inventors believed that the phenomena underlying rapid productive refolding by a method of the present invention was that secondary and tertiary structure of a protein was preserved even during application of elevated hydrostatic pressure. Because a "fully denatured" protein only retains primary structure, one skilled in the

art would have necessarily understood that the inventors were describing a method wherein protein aggregates were exposed to hydrostatic pressures in range of pressure which is insufficient to fully denature the protein (i.e., a pressure at which tertiary and/or secondary structure is retained).

6. I disagree with the Examiner's contention, regarding claims 1-6 lacking written description support, that exemplification of tailspike protein aggregates using the claimed methods, which the Examiner characterizes as a "a well-known protein in which the intermediate forms of the folding and aggregates pathway of the protein are known" is insufficiently representative of the claimed method to "to demonstrate that *applicant had possession of the full scope of the claimed invention.*" In particular, I disagree with the Examiner's contention that protein aggregates of tailspike folding intermediates would not convince a skilled artisan that protein aggregates of other proteins could be used in the method. I believe that the Examiner has overlooked the knowledge in the art as of October 25, 1999, the earliest date for which priority of the current application is claimed.

7. By 1998, the issue of protein aggregation as a topic of serious study in the protein folding art had already been been "elevated to almost cult status." **Exhibit 29**, p. 5253, col. 2, 2nd full paragraph. In particular, as detailed below, it was recognized in the art that: (1) protein aggregates comprise proteins having partially denatured conformations (i.e., proteins having at least secondary structure); (2) the predominant force mediating protein aggregation was intermolecular hydrophobic interactions; (3) hydrostatic pressure could be used to disrupt intermolecular interactions, and in particular, intermolecular hydrophobic interactions; and (4) proteins have an inherent tendency to refold properly if forces driving aggregation of folding intermediates are minimized or entirely disrupted.

8. Aggregation of native protein during processing and or storage of therapeutic protein formulations was (and still is) a common problem. However, it was well known in the art by 1998 at the latest:

   a. That protein aggregates can arise in protein formulations as a result of exposure to physical stresses (for example, shaking, mixing, thermal). See **Exhibits 1-9**.

   b. That the protein aggregates were formed in this context by noncovalent intermolecular interaction between partially denatured proteins (i.e., the protein generally retains secondary structural elements). See **Exhibits 1-2, 4-6** and **8**.

   c. And that protein aggregation in this context was generally driven by hydrophobic intermolecular interactions. See **Exhibits 1-2, 6** and **9**.

9. Aggregation of protein during recombinant synthesis (i.e., inclusion bodies) or purification of recombinant protein was (and still is) a common problem. In this regard, it was well known in the art by 1998 at the latest:

   a. That aggregation is a significant intermolecular side reaction that kinetically competes with intramolecular protein folding. See **Exhibit 10** (p. 13587, col. 2, ¶ 2; p. 13590, col. 2, last paragraph); **Exhibit 11**; **Exhibit 12**, p. 17067, col. 2, 2nd full paragraph (stating that:

> it is a common observation that the yield of renatured protein decreases when the concentration of the protein to be refolded increases. Kinetic competition between two types of interactions (interchain and intrachain) occurs during the refolding of a protein. Unimolecular intrachain interactions largely lead to the native state, while multimolecular interchain interactions would be expected to increase with the concentration of the refolding protein and therefore lead to misfolding and aggregation.);

Exhibit 13, p. 159, col. 1, 1st full paragraph; **Exhibit 14**, p. 5483, col. 1, 3rd full paragraph; and **Exhibit 15,** p. 3457, col. 1, end of 2nd full paragraph.

b. That protein aggregates (including inclusion bodies) generally comprise non-covalently associated protein folding intermediates. See **Exhibit 10,** p. 13587, col. 2, ¶ 2 and p. 13590, col. 2, 2nd full paragraph and last paragraph; **Exhibit 12**; **Exhibits 19-25**.

c. That the protein folding intermediates which form protein aggregates (including inclusion bodies) have considerable secondary structure. **Exhibit 13**, p. 159, col. 2, 1st full paragraph; **Exhibit 17**, sentence spanning pp. 1953-1954 and p. 1958, col. 2, 1st full paragraph; and **Exhibits 26-27**.

d. That protein folding intermediates tend to have an overall structure which exposes hydrophobic surfaces which possess a strong hydrophobic nature, the intermolecular hydrophobic interactions of which drive formation of protein aggregates. **Exhibit 10**, p. 13590, col. 2, 2nd full paragraph and last paragraph; **Exhibit 13**, p. 159, col. 2, 1st full paragraph – p. 160, col. 2, ¶ 1; **Exhibit 14**, p. 5483, col. 1, 3rd full paragraph (stating:

> It is most likely that the intermolecular association reflects specific interactions between hydrophobic surfaces of one partially folded molecule with those of another, where these specific interactions are ones that normally occur intramolecularly and lead to the formation of the native state.... In other words if we consider the monomeric native state to arise by the coalescence of structural building blocks (subdomains) in an intramolecular fashion, the aggregates arise by these same interactions but in an intermolecular fashion.);

Exhibit 16; **Exhibit 17,** p. 1958, col. 1, ¶ 1, last sentence; **Exhibit 18**; **Exhibit 23**; **Exhibit 28**, p. 428, col. 1, ¶ 2 (stating:

> It is now clear, based on investigations of transient and equilibrium intermediates in vitro, that partially folded intermediates, as found with newly synthesized proteins in the cell, are particularly prone to aggregate, probably via specific intermolecular interactions between hydrophobic surfaces of structural subunits. The intermediates are more prone to aggregate than the unfolded state because in the latter the hydrophobic side chains are scattered relatively randomly in many small hydrophobic regions, whereas in the partially folded intermediates, there will be large patches of contiguous surface hydrophobicity that will have a much stronger propensity for aggregation. The tendency of partially folded intermediates to associate or aggregate is exacerbated as the protein concentration increases. The growing recognition of the critical importance of protein aggregation has resulted in a number of reviews.

(citations omitted)).

10. Regarding the use of hydrostatic pressure to dissociate proteins from protein aggregates, as of the earliest date for which priority of the current application is claimed it was already recognized in the art by 1998 at the latest that:

a. Noncovalent interactions within a protein and between proteins can be reversibly perturbed using high hydrostatic pressure since protein folding and protein-protein interactions are normally accompanied by an increase in volume because of the combined effects of the formation of solvent-excluding cavities and the release of bound solvent. **Exhibit 30**, p. 9050, col. 2, ¶ 3; **Exhibit 35**, p. 1552, paragraph spanning columns 1-2 (stating:

> Our conceptual framework for pressure denaturation is as follows: the protein interior is largely composed of efficiently packed residues, more likely hydrophobic than those at the surface (19). Increasing hydrostatic pressure then forces water molecules into the protein interior, gradually filling cavities, and eventually breaking the protein structure apart.).

b. That subjection to elevated hydrostatic pressure causes dissociation of subunits of a multisubunit protein by interfering with hydrophobic interactions present in the

contacts between the subunits. **Exhibits 31-34**. See also p. 7, lines 18-22 of the current application.

c. That proteins subjected to elevated hydrostatic pressure can still retain elements of structural organization (i.e., secondary, and sometimes tertiary structure). **Exhibit 35**, p. 1552, col. 1, last paragraph. See also p. 7, line 23 – p. 8, line 3 of the current application.

11. Finally, it was already recognized in the art by 1998 at the latest that minimizing intermolecular interactions that lead to aggregation of partially folded intermediates can be an important factor in optimizing successful refolding. **Exhibit 10**, p. 13587, col. 2, ¶ 2 and p. 13590, col. 2, last paragraph; **Exhibit 12**, p. 17067, col. 2, 2nd full paragraph; **Exhibit 13**, p. 160, col. 1, last paragraph – col. 2, 2nd paragraph; and **Exhibit 18**. Indeed, as stated by Dobson, "Many studies have established that the vast majority of denatured protein chains are capable of refolding spontaneously to the correctly folded conformation in the absence of either other macromolecules or energy expenditure." **Exhibit 29**, p. 5251, col. 1, 1st paragraph after abstract.

12. In view of art recognized common attributes of protein aggregates in general (i.e., that they are comprised of incompletely folded protein predominantly driven to associate by hydrophobic intermolecular associations (see ¶¶ 8 and 9, above)), a skilled artisan would expect that elevated hydrostatic pressure could be used to dissociate protein from aggregates of other proteins, like the dissociation of protein from tailspike protein aggregates as exemplified in the current application.

13. Moreover, a skilled artisan would also be convinced that native protein could be recovered from protein which dissociates from protein aggregates subjected to hydrostatic pressure,

similar to the native tailspike recovered following application of hydrostatic pressure to tailspike protein aggregates, since the phenomena underlying the ability of a dissociated protein to assume a native conformation by the hydrostatic pressure methods of the present invention is no different than the phenomena relied upon by every other method of recovering native protein from protein aggregates (i.e., an inherent ability of incompletely folded protein to spontaneously refold to a native conformation).

14. In view of the teachings found in the application and the knowledge in the art as of October 25, 1999, it is my opinion that only routine experimentation would be required to determine whether protein aggregates of any particular protein are amenable for use in the currently claimed methods.

15. I note that the Examiner's discussion of the guidance set forth in the application as only including exemplification of tailspike protein aggregates (page 14 of the Office Action) ignores the general guidance found in the application at:

a. Page 3, lines 22-29, describing a general method for determining the optimal hydrostatic pressure to recover a native protein;

b. Page 10, lines 20-29, providing guidance on use of assays for monitoring native and non-native conformations, with specific exemplification of the use of HPLC in Example 1 of the application (pp. 14-17);

c. Page 11, lines 1-5, providing guidance on the use of reducing agents if the protein of interest contains post-translational modifications;

d. Page 11, lines 6-13, providing guidance on the use of chaperones or isomerases to assist refolding of a protein of interest; and

e. Page 12, ll. 15-27, providing guidance on the temperature at which hydrostatic pressure should be applied.

16. The Examiner's contention as to the lack of the guidance set forth in the application also ignores the extensive knowledge in the art regarding techniques for monitoring native and non-native conformations of proteins as evidenced by their use in the studies reported above. **Exhibits 1-35**. See also, **Exhibit 36**, pp. 445-446 ("Introduction"), and **Exhibit 37**, p. 2301, paragraph spanning columns 1-2.

17. Based on the guidance provided in the specification and the knowledge in the art in October, 1999, one skilled in the art would have been able to identify conditions under which hydrostatic pressure could be applied to cause dissociation of protein from protein aggregates comprising proteins other than tailspike using only a limited amount of experimentation.

18. Likewise, one skilled in the art in October, 1999 would also have been able to identify whether native protein was recovered following return of a sample to ambient pressure using assays, which while depending on the particular protein of interest, were conventional techniques used by skilled artisans in October, 1999.

19. Therefore, in my opinion, a skilled artisan in October, 1999, would have considered both the determination of the hydrostatic pressure conditions under which protein dissociates from protein aggregates as well as the determination of the presence of native protein following return to ambient pressure as routine experimentation. Extensive testing as to the suitability of other recovery methods or for optimizing recovery of protein from protein aggregates using other methods was common in the industry at that time. See **Exhibit 13**, page 161, paragraph spanning columns 1-2.

20. I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the above-identified application or any patent issues thereon.

Anne Skaja Robinson

Date 10/30/06

Application No. (if known): 10/673,000

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